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Phenazine-dye- and Enzyme-modified Plastic Formed Carbon Electrode for Amperometric Dihydronicotinamide Adenine Dinucleotide and Glucose Sensing

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1-Methoxy 5-methylphenazinium methyl sulphate (1-MeO-PMS) was adsorbed on the disk plane of a plastic formed carbon (PFC) rod electrode (3 mm in diameter). PFC is a novel carbon material prepared by mixing various amounts of pure graphite with an organic binder and pyrolyzing to "glassy carbon" at moderate final temperatures of 1000–1400 °C. The prepared 1-MeO-PMS/PFC electrode exhibited a sufficient electrocatalytic activity for dihydronicotinamide adenine dinucleotide (NADH) oxidation. At an applied potential of −0.05 V vs Ag/AgCl at pH 7.0, the 1-MeO-PMS/PFC electrode showed a fast (<5 s) and linear anodic current response to NADH over the concentration range of 1 to 100 μM with a limit of detection of 0.8 μM (S/N = 3). NAD^+-dependent glucose dehydrogenase (GDH) was further adsorbed on the 1-MeO-PMS/PFC electrode for glucose sensing. In the presence of 10 mM NAD^+ in 0.1 M phosphate buffer (pH 8.0), the prepared GDH/1-MeO-PMS/PFC electrode showed a fast (<10 s) and linear current response to glucose over the concentration range of 10 to 100 μM with a limit of detection of 7 μM (S/N = 3).

1. Introduction

Dihydronicotinamide adenine dinucleotide (NADH) is a key molecule in biological metabolic pathways, and many dehydrogenases use an oxidized coenzyme (NAD^+) and produce NADH concomitant with the oxidation of corresponding substrates. Therefore, the combination of the NAD^+-dependent dehydrogenases and NADH-sensing system allows the development of biosensors for many dehydrogenase substrates (e.g., glucose, lactate, glutamate, alcohol, and so forth).[1,2] From this viewpoint, the development of a sensitive, selective, and simple NADH-sensing system is desired. Among various analytical methods, electrochemical sensors are attractive because of their simplicity, low cost, high sensitivity, and wide applicability. However, the direct electrochemical oxidation of NADH at ordinal electrodes requires a large

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overvoltage,[1,3] which leads to unfavorable interferences from more easily oxidizable species in real samples (i.e., ascorbic acid and uric acid). In addition, the direct oxidation product of NADH causes electrode fouling, resulting in a low reproducibility.[1,4]

To solve these problems, the electrocatalytic oxidation of NADH with an appropriate mediator is one of the efficient ways.[5–17] Various redox-active compounds [e.g., organic dye,[7–13] quinone derivative,[14] and metal complex[15–17]] have been employed as mediators, and mediator-modified electrodes enabled the amperometric determination of NADH with a high reproducibility at a moderate detection potential.

Plastic formed carbon (PFC) is a novel carbon material that is prepared by mixing various amounts of pure graphite with an organic binder and pyrolyzing to "glassy carbon" at moderate final temperatures of 1000–1400 °C.[18,19] PFC has the following characteristics:[19] (i) Its preparation procedure allows the precise control of graphite adsorption characteristics and the reduction in diameter to a micrometer level. (ii) The crystal edge plane of graphite is located with a high density at the disk plane of PFC. (iii) The disk and cylindrical planes of PFC have microstructures similar to those of the edge and basal planes of the highly ordered pyrolytic graphite (HOPG), respectively. (iv) The PFC electrode shows a stable response of the surface reaction toward electroactive species with good reproducibility. (v) The PFC surface is almost as impermeable as a glassy carbon surface. (vi) The potential window on the PFC electrode corresponds to that on the glassy carbon electrode. On the basis of these features, the PFC electrode has been utilized in analytical fields, e.g., (a) as a precolumn adsorbent for liquid chromatography,[20] (b) as an electric transduction material for the study of cardiac muscle mechanics,[21] (c) as a working electrode of an electrochemical detector of HPLC,[22,23] and (d) as a base electrode for the direct electron transfer of myoglobin[24] and bilirubin oxidase.[25] However, few reports have been published regarding PFC-based chemical sensors and biosensors, except for several pioneering works [i.e., the direct electrochemical oxidation of NADH by bare PFC[26] and peroxidase- and ferrocene-embedded carbon-paste-modified PFC for H_2O_2 sensors and creatinine and creatine assays[27]]. In other words, the direct modification of a mediator and an enzyme on PFC, and its application to chemical sensors and biosensors have not been reported thus far to the best of our knowledge.

It is well known that aromatic organic compounds and proteins tend to strongly adsorb on the graphite surface by π–π stacking and hydrophobic interactions, respectively. Therefore, if redox-active aromatic compounds (mediators) and dehydrogenase enzymes can be effectively adsorbed on the PFC electrode and these adsorbed molecules keep their activities on the PFC surface, the development of PFC-based electrochemical NADH and enzyme sensors would be expected. It has been reported that various redox-active aromatic dyes (i.e., phenazine, phenoxazine, phenothiazine, and flavin derivatives) tend to strongly adsorb on the graphite electrode, and the resulting dye-modified electrodes exhibited the electrocatalytic activity for NADH oxidation.[5–13] Among many aromatic dyes, 1-methoxy 5-methylphenazinium methyl sulphate (1-MeO-PMS) is much more stable and strongly adsorbs on a pyrolytic graphite electrode.[7] Additionally, phenazine dye showed the highest rate constant as a mediator for the electrochemical oxidation of NADH as compared with p-quinone, 2,6,-dichlorophenol indophenol, methylene blue, and fravin adenine dinucleotide (FAD).[8]

Therefore, if 1-MeO-PMS strongly adsorbed on the PFC electrode and the adsorbed 1-MeO-PMS effectively works as a mediator for NADH oxidation, a mediator-modified PFC-based NADH sensor would be developed. Moreover, further immobilization of NAD^+-dependent dehydrogenase enables the development of an enzyme- and mediator-modified PFC electrode-based biosensor. In this study, 1-MeO-PMS was adsorbed onto the disk plane of the PFC electrode. As expected, the resulting 1-MeO-PMS/PFC electrode exhibited a sufficient electrocatalytic activity for NADH oxidation and a highly sensitive and rapid amperometric response to NADH at an applied potential of −0.05 V vs Ag/AgCl. Further adsorption of NAD^+-dependent glucose dehydrogenase (GDH) on the 1-MeO-PMS/PFC electrode enabled the development of an amperometric biosensor for the sensitive and rapid determination of glucose. In addition to a very simple and rapid preparation procedure, the volume of the mediator and enzyme solution for immobilization is quite small (5 μl). Furthermore, the fresh disk plane of the PFC rod can easily be regenerated by polishing with emery paper and exhibits reproducible electrochemical responses. Therefore, as compared with other carbon-material-based biosensors, the repeated preparations are quite easy with a low cost and a short time. Moreover, the proposed system would be applicable to various kinds of NAD^+-dependent dehydrogenases.

2. Materials and Methods

2.1 Reagents and materials

A PFC rod (3 mm in diameter, 50 mm in length) was donated by Mitsubishi Pencil Co. 1-MeO-PMS was purchased from Sigma-Aldrich Co. NADH, NAD^+, and D-glucose were obtained from Wako Pure Chemical Industry. NAD^+-dependent GDH (EC. 1.1.1.47, from microorganism, 250 unit/mg) was obtained from Toyobo Co. and used without further purification. All of the other chemicals were of the highest grade available. The 0.1 M phosphate buffer solution prepared with K_2HPO_4 and KH_2PO_4 was used as the electrolyte. Millipore Milli-Q water (resistivity > 18 MΩcm, TOC, 3 ppb) was used for the preparation of all solutions throughout the experiments.

2.2 Preparation of NADH- and glucose-sensing electrodes

The disk plane of the PFC rod was polished with #800 emery paper and washed by ultrasonication in pure water for 5 min. The side wall of the PFC rod was shielded with a parafilm except for 10 mm from the top for electric contact toward the analyzer. For the preparation of the 1-MeO-PMS/PFC electrode, 5 μl of 5 mM 1-MeO-PMS aqueous solution was placed on the disk plane of the PFC electrode and allowed to stand for 5 min at room temperature (25 ± 2 °C) for the adsorption of 1-MeO-PMS. Subsequently, the 1-MeO-PMS-adsorbed PFC (1-MeO-PMS/PFC) electrode was rinsed with water and placed in a fresh buffer solution for 30 min before use. For the preparation of the GDH/1-MeO-PMS/PFC electrode, 5 μl of GDH (0.5 mg/ml)-containing 0.1 M phosphate buffer (pH 8.0) was placed on the disk plane of the 1-MeO-PMS/PFC electrode and allowed to stand for 2 h at room temperature to adsorb

GDH. The resulting GDH/1-Me-O-PMS/PFC electrode was rinsed with water and placed in 0.1 M phosphate buffer (pH 8.0) at 4 ℃ for 1 h before the electrochemical measurement.

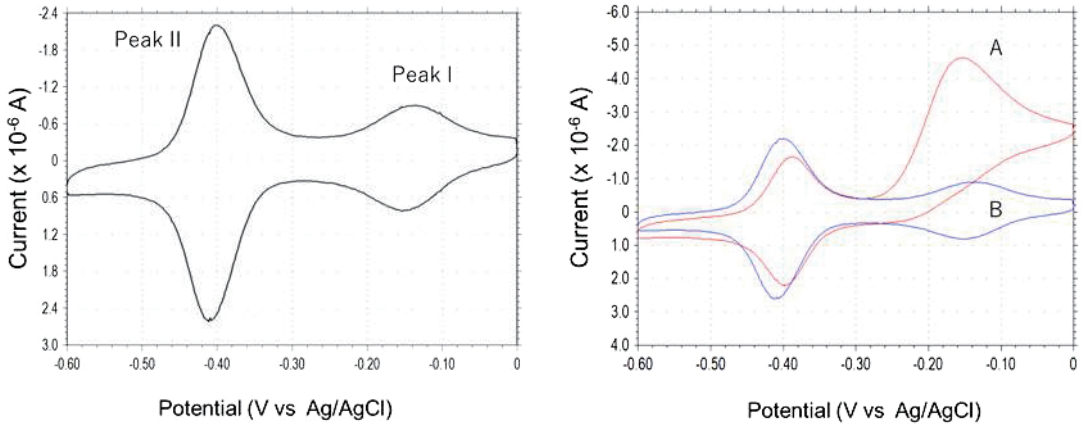
2.3 Electrochemical measurement

Electrochemical measurement was carried out with a conventional three-electrode system by using an electrochemical analyzer (ALS 611B). 1-MeO-PMS/PFC or GDH/1-MeO-PMS/PFC electrodes were used as working electrodes. A Pt wire (1 mm in diameter) was used as a counter electrode. A Ag/AgCl electrode (BAS, RE-1B) was used as a reference electrode. Cyclic voltammetry (CV) was carried out using the deoxygenated 0.1 M phosphate buffer prepared by bubbling N_2 into the electrolyte for at least 20 min. During the CV measurement, N_2 atmosphere was kept by the gentle flow of N_2 on the upper part of the air/solution interface of the electrolyte. Constant potential amperometry was conducted with an applied potential of −0.05 V with gently stirred air-saturated 0.1 M phosphate buffer (pH 7.0 for NADH detection and pH 8.0 for glucose detection, because the optimum pH of the present GDH is pH 8–9). After the background current reached steady-state values, different concentrations of the standard solution of NADH or glucose were added every 40 s, and current-time curves were recorded. For NADH sensing, the 1-MeO-PMS/PFC electrode was kept in 0.1 M phosphate buffer (pH 7.0) at 4 ℃ in a refrigerator between measurements. For glucose sensing, the GDH/1-MeO-PMS/PFC electrode was kept in 0.1 M phosphate buffer (pH 7.0) at 4 ℃ in a refrigerator between measurements because GDH shows a maximum pH stability at 7.0.

3. Results and Discussion

3.1 Electrochemical properties of 1-MeO-PMS/PFC electrode

It has been reported that 1-MeO-PMS strongly adsorbs on the basal plane of a pyrolytic graphite pyrolytic graphite (PG) electrode and that the 1-MeO-PMS/PG electrode shows reversible voltammetric responses.[7] Additionally, the adsorbed 1-MeO-PMS is stabilized by adsorption.[7] Because PFC has strong adsorption properties toward various aromatic organic compounds, a similar redox property can be expected on the 1-MeO-PMS/PFC electrode. Figure 1(a) shows a cyclic voltammogram of the 1-MeO-PMS/PFC electrode obtained in deoxygenated 0.1 M phosphate buffer (pH 7.0). The 1-MeO-PMS/PFC electrode exhibited two redox peaks. The redox wave observed in a more positive region ($E^{0'}$ = −0.147 V) originates from the adsorbed 1-MeO-PMS, and that observed in the negative region ($E^{0'}$ = −0.405 V) originates from the decomposed species (i.e., phenazine without methyl sulphate; 1-MeO-P).[7] The surface coverage (Γ) values of the adsorbed 1-MeO-PMS and 1-MeO-P were estimated to be ca. 2.8×10^{-10} and 1.1×10^{-9} mol/cm^2, respectively, using $Q = nFA\Gamma$, where n is the charge of the redox reaction (in this case, $n = 2$), Q is the quantity of electricity of the anodic wave, F is the Faraday constant, and A is the area of the electrode surface (in this case, $A = 0.07$ cm^2; geometric area). These values are close to those for the adsorbed PMS and P on the pyrolytic graphite electrode.[7]



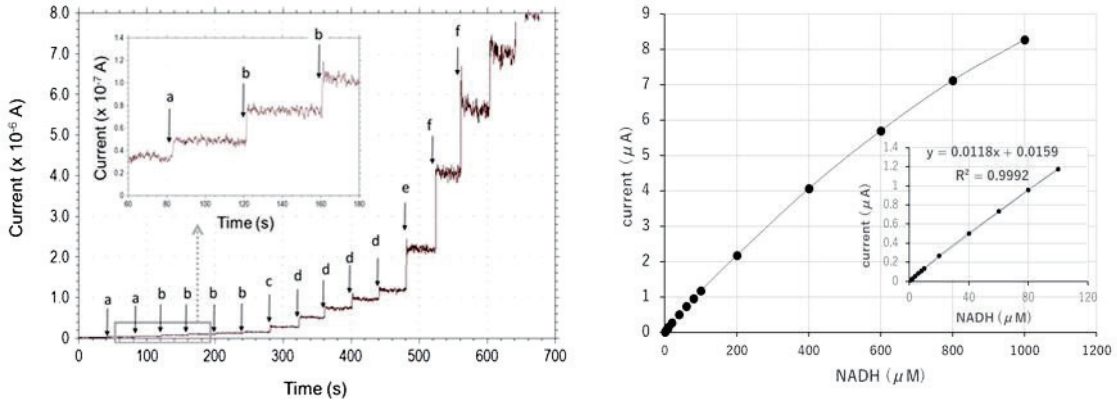
(a) (b)

Fig. 1. (Color online) (a) Cyclic voltammogram of 1-MeO-PMS/PFC electrode in deoxygenated 0.1 M phosphate buffer (pH 7.0). The starting potential is −0.6 V. The potential scan rate is 5 mV/s. (b) Cyclic voltammograms of 1-MeO-PMS/PFC electrodes (A) with and (B) without 1 mM NADH in deoxygenated 0.1 M phosphate buffer (pH 7.0). The CV measurement condition is the same as that in panel (a).

Figure 1(b) shows a cyclic voltammogram of the 1-MeO-PMS/PFC electrode in the presence and absence of 1 mM NADH. In the presence of NADH, the anodic current of Peak I increased and the cathodic current disappeared (A, red curve). In contrast, the shape of Peak II almost did not change even in the presence of NADH. These results indicate that the adsorbed PMS has an efficient electrocatalytic activity for NADH oxidation, whereas the decomposed P does not have the electrocatalytic activity. This tendency is also similar to the case of the 1-MeO-PMS/pyrolytic graphite electrode.[7] Here, the oxidation peak potential of NADH on the 1-MeO-PMS/PFC electrode is −0.15 V, while that on the bare PFC is 0.35 V (data not shown), indicating that the 1-MeO-PMS/PFC electrode lowers the NADH oxidation peak potential by 400 mV through the mediated reaction.

3.2 Amperometric response of NADH obtained using 1-MeO-PMS/PFC electrode

Next, we evaluated the NADH-sensing property of the 1-MeO-PMS/PFC electrode by constant potential amperometry. Figure 2(a) shows the steady-state anodic current responses toward the successive addition of NADH at −0.05 V vs Ag/AgCl. We selected −0.05 V to reduce the background current originating from the electrochemical reduction of oxygen dissolved in the stirred electrolyte. The 1-MeO-PMS/PFC electrode showed a clear response even to 1 μM NADH [Fig. 2(a) inset], and the response time for 95% of the steady-state current was less than 5 s. Amperometric noise increased in the high NADH concentration region. It has been reported that the electrode capacitance and resistance of the electrochemical cell influence current noise when using constant-potential amperometry.[28] Because NAD^+ and NADH are reported to adsorb on the carbon electrode surface,[29] the observed noise would originate from the change in electrode capacity by these adsorptions. As shown in Fig. 2(b), the calibration curve was linear over the NADH concentration range from 1 to 100 μM with a



(a) (b)

Fig. 2. (a) Current-time curve of 1-MeO-PMS/PFC electrode for successive additions of different concentrations of NADH at −0.05 V vs Ag/AgCl. The electrolyte is air-saturated 0.1 M phosphate buffer (pH 7.0) subjected to gentle stirring. The inset shows an enlargement of the low NADH concentration region. NADH concentrations: a, 1; b, 2; c, 10; d, 20; e, 100; and f, 200 μM. (b) Calibration curve of NADH obtained using 1-MeO-PMS/PFC electrode. The inset shows an enlarged linear range of the calibration curve.

regression coefficient of 0.9992. The sensitivity was 0.118 μA/μM, and the limit of detection was found to be 0.8 μM (S/N = 3, noise level = 5 nA). The reproducibility of the response to the same concentrations of NADH was good, and the relative standard deviation (RSD) (n = 5) varied from 0.56 to 1.88. A comparison of the analytical characteristics of the 1-MeO-PMS/PFC electrode and other NADH-sensing electrodes is shown in Table 1. By employing 1-MeO-PMS as the mediator, the detecting potential can be shifted to a negative value of more than 400 mV as compared with the case of the bare PFC.[26] The sensitivity of the 1-MeO-PMS/PFC electrode is superior to those of other organic-dye-modified electrodes.

The effect of the adsorption time of 1-MeO-PMS on the sensitivity of the sensor was investigated. Surprisingly, the sensitivity of the sensor prepared by 5 min adsorption (0.118 μA/μM) was not markedly different from those of the sensors prepared by 1 h (0.111 μA/μM) and 24 h (0.099 μA/μM) adsorption processes, suggesting that a stable adsorbed layer of 1-MeO-PMS on the disk plane of PFC is formed in a short time, and that the prolonged adsorption period is not required to create an effective catalytic layer of 1-MeO-PMS on the PFC electrode. When a glassy carbon electrode was used in place of PFC, no apparent redox peaks and responses to NADH were observed under the same experimental conditions. The validity of each 1-MeO-PMS/PFC electrode was examined using four different electrodes. The fabrication reproducibility was evaluated from the sensitivity (slope of the linear portion), and the RSD (n = 4) was evaluated to be less than 8%.

3.3 Amperometric response of glucose obtained using GDH/1-MeO-PMS/PFC electrode

NAD^+-dependent dehydrogenases convert NAD^+ to NADH in the presence of an appropriate substrate. In this study, we employed GDH as a model enzyme and investigated the possibility of the development of PFC-based amperometric biosensors for glucose as our preliminary

Table 1
Comparison of analytical performance characteristics of NAD^+-dependent GDH-based glucose sensors.

Electrode	Mediator	Detecting potential (V vs Ag/AgCl)	Linear range (μM)	Limit of detection (μM)	Ref.
PFC	1-MeO-PMS	−0.05	1–100	0.7	This work
PFC	none	+0.4	50–1000	—	26
PG	Disperse blue	+0.15	2–100	2	9
CNT/GC	Meldola's blue	−0.1	up to 500	0.048	10
CNT/PG	Toluidine blue	−0.2	50-500	5	11

PG, pyrolytic graphite; CNT, carbon nanotube; GC, glassy carbon

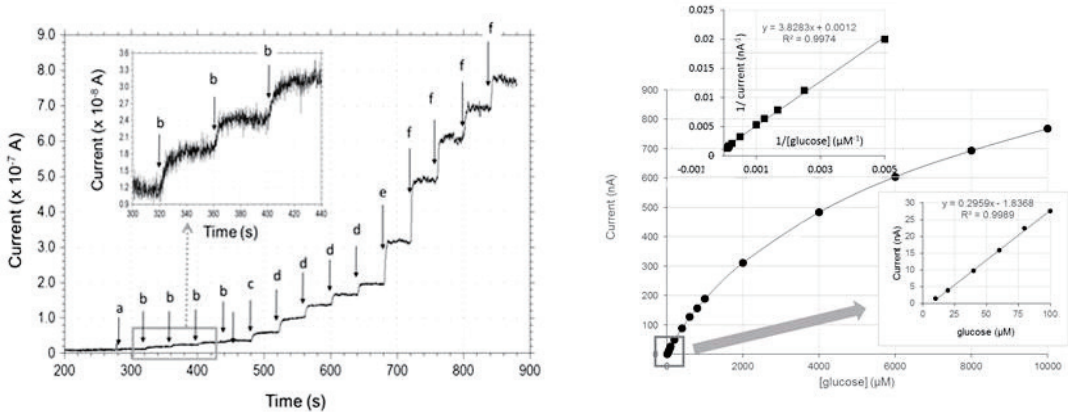


Fig. 3. (a) Current-time curve of GDH/1-MeO-PMS/PFC electrode for successive additions of different concentrations of glucose at applied potential of −0.05 V vs Ag/AgCl. The electrolyte is air-saturated 0.1 M phosphate buffer (pH 8.0). The inset shows an enlargement of the low glucose concentration region. Glucose concentrations: a, 10; b, 20; c, 100; d, 200; e, 1000; and f, 2000 μM. (b) Calibration curve of glucose obtained using GDH/PMS-PFC electrode prepared on the basis of the responses in panel (a). The lower inset shows an enlarged linear range of the calibration curve. The upper inset shows a Lineweaver–Burk plot.

approach. Figure 3(a) shows a typical current-time curve for the sensor when the glucose concentration increased (40 μl of appropriate glucose standard solution was successively added to 15 ml of electrolyte after a constant background measurement had been established at the points indicated by the arrows). The steady-state current changed rapidly upon the addition of glucose and reached another steady-state current within 5–10 s. Figure 3(b) shows the calibration curve of glucose obtained using the GDH/1-MeO-PMS/PFC electrode. A linear relationship was obtained at concentrations ranging from 10 to 100 μM with a sensitivity of 0.2959 μA/μM and a correlation coefficient of 0.9989. The fabrication reproducibility was evaluated from the sensitivity, and the RSD (n = 3) was less than 10%. The electrochemical Lineweaver–Burk double reciprocal plot is given by

$$1/I = (K_m^{app}/I_{max})(1/[\text{glucose}]) + 1/I_{max}, \quad (1)$$

where I is the steady-state current, I_{max} is the maximum current under stationary substrate conditions, and K_m^{app} denotes the apparent Michaelis constant. The upper inset of Fig. 3(b)

Table 2
Comparison of analytical performance characteristics of NAD^+-dependent GDH-based glucose sensors.

Electrode	Mediator	Linear range (mM)	Limit of detection (μM)	Ref.
PFC	1-MeO-PMS	0.01–0.1	7	This work
SPC	Meldola's blue	0.075–30	75	12
CNT/GC	Poly(Nile blue)	0.1–8.5	50	13
SPC	Fe(III) complex	1.7–33	666	16
CNT paste	Os(II) polymer	up to 0.8	10	17

SPC, screen-printed carbon electrode; CNT, carbon nanotube

shows a Lineweaver–Burk plot, where the inverse of the response current is plotted against the inverse of the glucose concentration. K_m^{app} and I_{max} were estimated to be 3.19 mM and 833 μA, respectively, using the equation $Y = 3.8283X + 0.0012$ ($r^2 = 0.9989$). The obtained K_m^{app} value (3.19 mM) is much smaller than that for GDH in solution (13.8 mM),[30] indicating that the immobilized GDH on the PFC electrode has an increased affinity to the glucose. This feature is desirable for the highly sensitive determination of glucose by the GDH/1-MeO-PMS/PFC electrode.

Table 2 shows a summary of the analytical performance characteristics of NAD^+-dependent GDH-based glucose-sensing electrodes. The present GDH/1-MeO-PMS/PFC electrode showed the highest sensitivity to glucose. This excellent sensitivity and fast response time (<10 s) suggest that immobilization on the PFC electrode surface does not appreciably affect enzyme activity, and glucose and NADH are not hindered from reaching the enzyme and electrode surface, respectively. Although the linear range is narrow (0.01 to 0.1 mM) in this stage, the GDH/1-MeO-PMS/PFC electrode showed a relatively wide dynamic concentration range of 0.01 to 10 mM [Fig. 3(b)]. Therefore, the present narrow linear range would be improved by further optimization of sensor preparation conditions (e.g., optimization of enzyme loading and coating of the outermost layer with an appropriate polymer film). Unfortunately, the operational and storage stabilities of 1-MeO-PMS/PFC and GDH/1-MeO-PMS/PFC electrodes are not sufficient in this stage (the sensitivities of NADH and glucose both decreased by less than half during 10 repetitive operations and/or after 1 d of storage in buffer). A more detailed optimization of the adsorption conditions (e.g., concentrations of 1-MeO-PMS and GDH, adsorption solvent, adsorption time, and adsorption pH) and another approach (e.g., chemical modification of the enzyme on the PFC surface and employing appropriate polymer supports) would be effective for improving both operational and storage stabilities, and these approaches are now under way.

4. Conclusions

In this study, we developed simple and relatively highly sensitive NADH and glucose sensors using a PFC rod electrode as a base signal transduction material. 1-MeO-PMS was adsorbed on the disk plane of the PFC rod electrode, and the adsorbed PMS exhibited a sufficient electrocatalytic activity for NADH oxidation. The resulting 1-MeO-PMS/PFC electrode exhibited rapid and sensitive amperometric responses to NADH at −0.05 V vs Ag/AgCl. By further adsorption of NAD^+-dependent GDH on the 1-MeO-PMS/PFC electrode, the rapid and

sensitive amperometric determination of glucose was possible. This methodology would lead to future wide applications of PFC as a base matrix of electrochemical biosensors and biofuel cells.

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